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14. ABSTRACT

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Dynamic Cyclic Thiodepsipeptide Libraries From Thiol-Thioester Exchange

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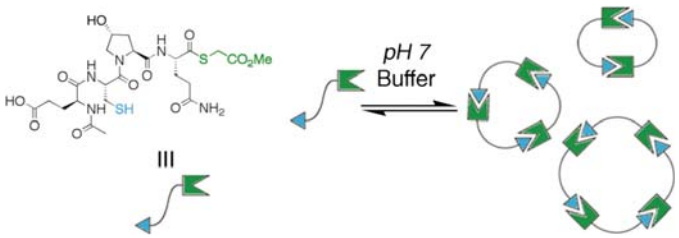
ABSTRACT



Thiol−thioester exchange was found to readily generate libraries of cyclic thiodepsipeptides under thermodynamic control, which will enable their use in a variety of dynamic combinatorial chemistry assays. The kinetic determinants of macrocycle formation and the role of amino acid structure on the reaction dynamics are discussed.

Cyclic peptides have been described as "privileged structures" for drug design because so many natural and synthetic cyclic peptides exhibit biological acitivity.[1−3] For example, cyclosporin is a cyclic nonribosomal peptide that is widely used as an immunosuppressant, revolutionizing organ transplants.[1] As another example, both natural and synthetic cyclic peptides have recently been identified as HDAC inhibitors, which hold promise for treatment of cancer.[4] As drug scaffolds, cyclic peptides are advantageous because they mimic native protein structure, exhibit enhanced metabolic stability, and are structurally preorganized, which reduces the entropic cost of binding.

Despite their therapeutic potential, the options for synthesizing structurally diverse libraries of cyclic peptides in a high-throughput format are limiting.[3] For example, phage display of disulfide-linked cyclic peptides has been used to screen large libraries of potential inhibitors,[5] but disulfides are not stable within the cellular environment and there is no straightforward synthetic replacement for a disulfide linkage. Split-and-pool synthesis is another popular method of generating libraries of cyclic peptides but requires orthogonal protecting group strategies of on-bead cyclization and can result in mixtures of cyclic and acyclic peptides.[3]

Herein, we describe a strategy to use thioester exchange for generating solution-phase cyclic thiodepsipeptide libraries via dynamic combinatorial chemistry (DCC).[6] DCC is a method for the in situ generation of a complex mixture of macrocycles from smaller building blocks via reversible covalent bond formation. In DCC, the members of an equilibrating library compete for an added template and

[†] U.S. Army Research Office.

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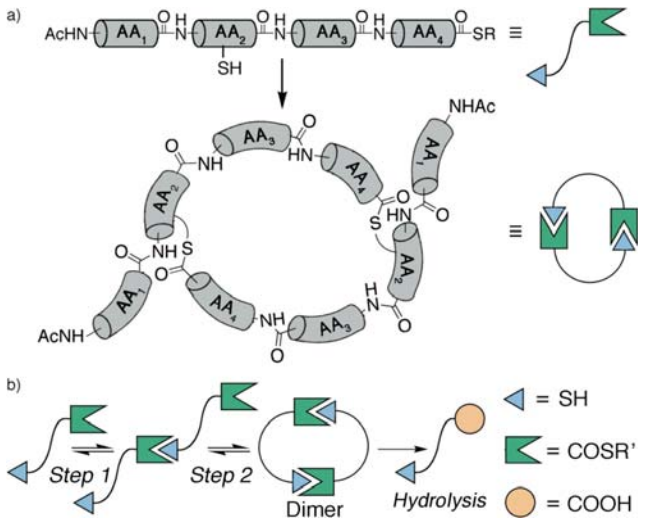

Figure 1. (a) General design of monomers and major products. (b) Sequence of steps toward dimeric cyclic thiodepsipeptide.

respond to amplify the best binder if one exists. Recent reports suggest that thiol-thioester exchange is a promising reversible reaction for DCC since it is rapid in aqueous solution at neutral pH[7,8] and provides a native-like linkage that is subsequently replaceable by more robust amide or ester functionalities. However, all reported cases of thioester exchange in DCC used thioesters that were unsubstituted at the α-position, which would significantly limit the diversity of structures possible in a library of cyclic peptides. Thus, we have investigated the reactivity of peptidic thiol-thioester exchange to determine the scope and limitations for its application to DCC.

Figure 1a shows the general structure of the monomers used in this study. Each monomer was a four-residue peptide containing a thioester at the C-terminus and a thiol at AA_2 (Cys). Structural variations were then made in AA_1, AA_3, and AA_4 (Table 1); by design, $AA_2-AA_3-AA_4$ forms the macrocycle, and AA_1 remains exocyclic. Charged amino acids (Lys, Arg, Glu) were incorporated into position AA_1 to enhance water solubility and prevent aggregation. Proline (or hydroxyproline, Hyp, to improve solubility) was initially included in AA_3 as a turn residue that favors macrocycles.[9] Various amino acids were incorporated into AA_4 to study their effect on macrocyclization, including positively charged amino acids (Lys, Arg), a negatively charged amino acid (Glu), a hydrogen-bonding and neutral amino acid (Gln), and hydrophobic and sterically bulky amino acids (Phe, Val).

The monomers were synthesized using a modification of the method reported by Gellman and co-workers (Supporting Information),[8] which provided monomers **1**−**15** in 70−80% yield with no observable epimerization. Upon dissolving **1**−**15** in potassium phosphate buffer (pH 7), the monomers underwent thiol−thioester exchange to form a mixture of macrocycles, with the dimeric 20-atom macrocyclic hexapeptides as the major product (Figure 1a). Based on HPLC analysis of the reaction mixture over time (Figure 2), we propose the simple two-step mechanism shown in Figure 1b. First, two monomers react to form an oligodimer followed by the ring closing intramolecular trans thioesterification reaction. The oligodimer intermediate was not observed to accumulate, indicating that the intermolecular step is rate limiting.[10]

Not surprisingly, the rate of macrocycle formation was dependent on the amino acid sequence. Since the first step in Figure 1b is rate determining, the overall reaction rate was determined by monitoring the disappearance of monomer over time, which in turn was measured by manual integration

Table 1. Monomers, Half-Life of Reactions, And Conversions of **1**−**15** (100 mM NH_4Ac Buffer, pH 6.75, 25 °C)

compd	sequence	$t_{1/2}$	product
1	Ac-ECHyp**G**-COSR′	~12 min	dimer
2	Ac-ECP**H**-COSR′	~12 min	dimer, trimer
3	Ac-ECHyp**F**-COSR′	~1.5 h	dimer
4	Ac-EC**P**F-COSR′	~1.5 h	dimer
5	Ac-ECHyp**Q**-COSR′	~1.5 h	dimer, trimer, tetramer
6	Ac-ECHyp**E**-COSR′	~5.5 h	dimer
7	Ac-ECHyp**V**-COSR′	ND[a]	oligodimer
8	Ac-EC**W**E-COSR′	~3.5 h	dimer
9	Ac-EC(**D-P**)Q-COSR′	~10 min	dimer, trimer
10	Ac-**K**CP**R**-COSR′	~15 min	dimer
11	Ac-**R**CP**K**-COSR′	~15 min	dimer
12	Ac-**K**CP**K**-COSR′	~15 min	dimer
13	Ac-KCP**Q**-COSR′	~20 min	dimer, trimer
14	Ac-KC**W**R-COSR′	~15 min	dimer
15	Ac-KCP**V**-COSR′	~10 h	dimer, trimer

[a] Not determined. Monomers were hydrolyzed before complete reaction.

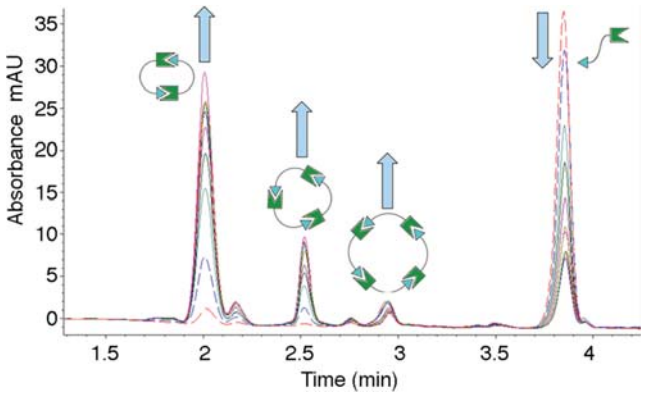

Figure 2. Overlay of HPLC traces showing the consumption of **5** and the appearance of macrocycles over time.

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(10) The macrocyclic thiodepsipeptides were found to hydrolyze over extended periods, but not before reaching equilibrium.

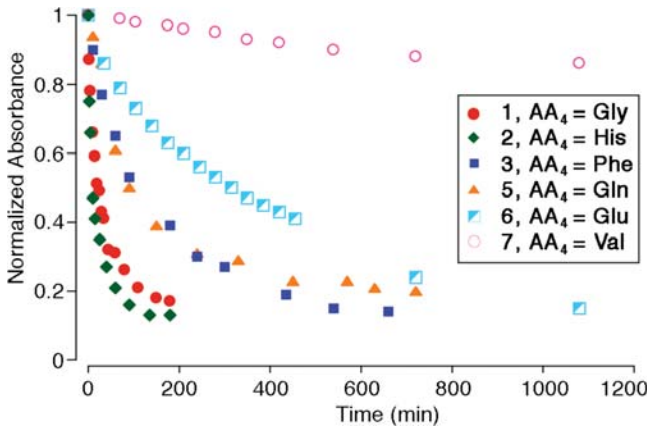

Figure 3. Plot of the disappearance of monomers **1**−**3** and **5**−**7** over time (100 mM NH_4Ac buffer, pH 6.75, 25 °C).

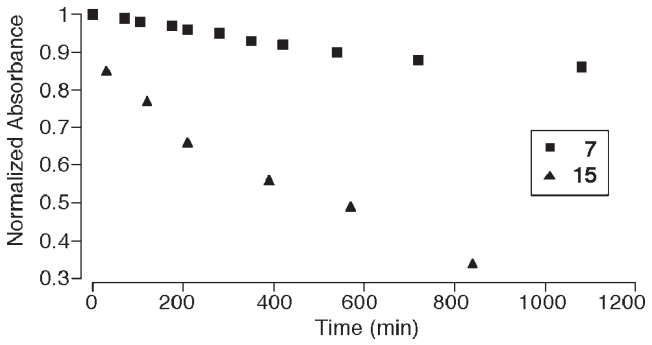

Figure 4. Comparison of the disappearance of monomers **7** and **15** over time (100 mM NH_4Ac buffer, pH 6.75, 25 °C).

of the area under the monomer peak in the HPLC−UV trace. Since several cases were too fast to measure at pH 7, the reactions were followed at pH 6.75 for comparison. Importantly, with the exception of monomers containing Val at the C-terminus, equilibrium was always reached prior to the detection of hydrolysis, and no acylation of Lys was observed.

Initially, the C-terminal amino acid (AA_4) was varied while holding the rest of the molecule constant (Table 1, compounds **1**−**7**). The observed reactivity trend, **1** ∼ **2** > **3** ∼ **4** ∼ **5** > **6** > **7**, corresponds generally to differences in steric effects at the C-terminal amino acid, but in some cases, the differences in rate are more subtle (Figure 3, Table 1). This trend is generally consistent with the reactivity trends observed in the native chemical ligation of peptides and sugar-assisted ligation of glycopeptides, which initiate with the same first step.[11,12] We find that His reacts as rapidly as Gly (Table 1, **1** vs **2**), despite its larger size, as was observed by Dawson,[11] suggesting that the imidazole group of the C-terminal His stabilizes the transition state of the trans thioesterification reaction. In contrast, β-branched amino acids such as Val react significantly more slowly (Table 1, entry **7**, and Figure 3), resulting in measurable hydrolysis before cyclization was complete. Monomer **6**, which contains Glu at position 4, reacts more slowly than its Gln analogue and forms multiple mass degenerate isomers with different retention times in the HPLC/MS. On the basis of the mass spectrometric analysis of individual peaks and literature precedent, it appears that the γ-carboxyl cyclizes onto the thioester to form anhydride and ultimately **6-isom** (see the Supporting Information).[13]

We varied several other positions to further explore reactivity. Replacement of Hyp with Pro resulted in identical HPLC traces and rate profiles for **3** versus **4**, indicating that the OH group on hydroxyproline does not affect the rate of reaction. We also investigated the replacement of Hyp with the more flexible Trp (monomer **8**) to determine if a turn is necessary for rapid cyclization. In fact, we found that monomer **8** reacts faster than the Hyp analogue. Stereochemistry was also a factor in the reaction rates as **9** (D-Pro) reacted considerably faster than **5** (Hyp). This result does not reflect conformational preferences for cyclization but instead an influence of chirality on the accessibility of the thioester or thiol.

We also investigated the effect of positively charged amino acids (Lys and Arg) at positions AA_1 and AA_4. Interestingly, monomers **10**−**15**, which contain Lys or Arg at AA_1 and/or AA_4, react significantly faster than their negatively charged analogues (Supporting Information). This rate enhancement suggests that the positively charged Arg and Lys residues stabilize the buildup of negative charge in the transition state through hydrogen bonding and/or electrostatic interactions. To further explore the influence of positively charged amino acids on reactivity, we replaced the Glu at AA_1 of monomer **7** with Lys to give monomer **15**. Although hydrolysis was still observed before a complete cyclization, **15** reacts almost five times faster than **7** (Figure 4).

When we analyzed binary mixtures of monomers **1**−**7** and **10**−**15** by HPLC, we observed the formation of a mixture of macrocyclic thiodepsipeptides with homo- and heterodimers as the major products. To confirm thermodynamic control over the library speciation, experiments were performed adding **1** to the preformed homodimer $\mathbf{3_2}$ and **3** to the preformed homodimer $\mathbf{1_2}$. Nearly superimposable HPLC traces were obtained after 18 h at pH 6.75, indicating that the equilibrium position was reached from both directions.

Figure 5 demonstrates that high chemical diversity is achievable upon simple mixing of monomers, in this case 1 h after mixing **1**, **3**, and **5**−**7**. It is worth noting that **7** was well incorporated in the library despite its slow reaction with itself. In addition to the cyclodimers, cyclo-tripeptides were present. The library speciation was readily deconvoluted using LCMS, which showed extensive cross-reactivity of monomers and a general lack of self-sorting.[14]

In summary, we have demonstrated an efficient methodology to rapidly generate a complex library of macrocyclic

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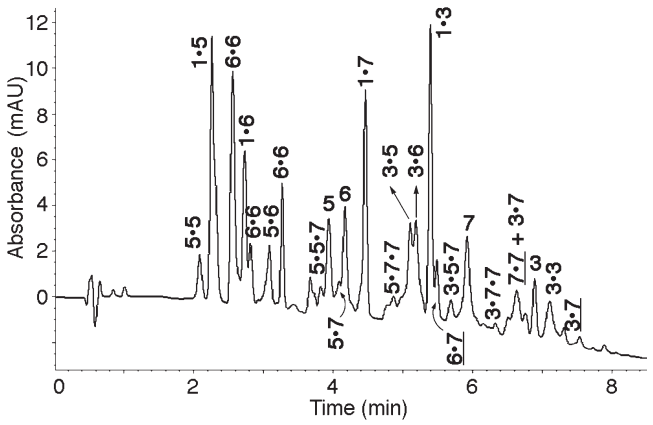

Figure 5. HPLC trace of a mixture of **1**, **3**, and **5**−**7** after equilibration for 1 h (100 mM NH_4Ac buffer, pH 6.75, rt). Underlined species indicate linear oligomers.

thiodepsipeptides at neutral pH for high-throughput screening. Structure−function studies indicate that the thiol-thioester exchange is tolerant of a variety of amino acids at the C-terminal position, with the exception of β-branched amino acids such as Val, which retard the rate of homodimerization, and carboxylic acid side chains, which can isomerize through anhydride intermediates. Positively charged amino acids and His at the C-terminus were found to enhance the rate of thiol-thioester exchange, suggesting a stabilizing influence through hydrogen-bonding and/or electrostatic interactions on the tetrahedral intermediate. Interestingly, positively charged amino acids were also found to increase reaction rates when incorporated at the N-terminus, suggesting that the transition state can be stabilized when flanked by a positively charged amino acid on either side. In all cases, the dimer is the major product, but the degree of formation of larger macrocycles is dependent on the peptide sequence. Upon mixing two or more monomers, we have shown that a complex library of cyclic thiodepsipeptides can be generated in situ under thermodynamic control.

This study demonstrates the feasibility of this methodology for generating a large number of cyclic thiodepsipeptides that can be screened against a particular target efficiently within hours. Since thioesters have transient stability in vivo, a "hit" in the high-throughput screening can be resynthesized as a more stable analogue by replacing of the thioester group with an amide or ester. Moreover, upon mixing different monomers containing Cys located at different positions, macrocycles of different sizes can in theory be generated, such that the outcome is not limited to only cyclic hexapeptides. In the future, we aim to apply this high-throughput screening method for inhibitor development, disruption of protein−protein interactions, and binding of nucleic acids.

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Supporting Information Available: Synthetic procedure, characterization data, and HPLC traces. This material is available free of charge via the Internet at http://pubs.acs.org.

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